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ABSTRACT

Colorectal carcinoma (CRC) is one of the main public health problems. The mortality of CRC is about 8%. Early detection of CRC is very important to prevent death because this cancer could be cured through surgery if the diagnosis can be made as early as possible. Therefore screening strategy for early detection of CRC is critical in reducing mortality. Many investigations supporting the detection of CRC have been developed, including the fecal DNA mutation test using advanced cytological techniques. It is capable of assessing colonocytes for the presence of DNA, RNA, and protein as molecular biomarkers of neoplasia in CRC, including p53 and hMLH1. This study implemented observational approach with a cross-sectional study of the feces of patients with CRC regardless of the stage and grade. The purpose of this study was to determine the expression of the hMLH1 and p53 mRNA genes in the feces of 48 patients with CRC from two hospitals in Indonesia, Siloam Hospitals in Cikarang and Dr. Wahidin Sudirohusodo Hospital in Makassar. The results showed that all adenocarcinoma feces samples with various tumor stages and grades had excess mRNA expression (more than twice the normal amount in Fold Change units) for both the hMLH1 and p53 genes. The average expression of the hMLH1 mRNA gene was the highest at stage two and grade one, while the lowest was at stage four and grade three. In contrast, the average p53 mRNA gene expression was the highest at stage four and grade three, while the lowest was at stage two and grade one. The study suggested that there was a relation between and and the expression of hMLH1 and p53 mRNA gene . . We concluded that while both hMLH1 and p53 genes in patients' feces with CRC were overexpressed, they did not significantly affect the grade of CRC.

Keywords: hMLH1, p53, Colorectal Carcinoma

Research Registry : researchregistry7319

Ethical Approval : 798/UN4.6.4.5.31/PP36/2020 (8 December 2020)

1. Background

Colorectal carcinoma (CRC) is one of the main public health problems and concerns, both in developed and developing countries (1). The frequency of CRC ranks third in both men and women, and the mortality is about 8%. CRC screening strategy plays an important role in preventing death and reducing CRC because this cancer can be cured through surgery if an appropriate diagnosis is taken place as early as possible. There are available methods to investigate CRC including Fecal Occult Blood Tests (FOBT) which are non-invasive and very useful with sigmoidoscopy or colonoscopy (2). However, the tests haverelativelyhigh false-positive rate arenonspecific, and not sensitive enough for early-stage CRC detection, even though high sensitivity results were reported in the advanced stage of CRC (2) .

A study by Dong *et al*. identified the combination of p53, BAT 26, and K-ras gene mutations in feces in 71% of colorectal carcinoma patients (3). Ahlquist *et al*. improved the detection sensitivity to 91% using a panel consisting of the same genes with an addition of one more gene, APC, to the combination (4). It has also been acknowledged that RNA-based detection methods are more comprehensive than DNA, protein, or methylation-based methods (5, 6).

Three major alteration can occur in CRC, namely microsatellite instability (MSI), chromosomal instability (CIN), and CpG island methylator phenotype (CIMP) which modify the DNA, RNA, protein, or metabolites, which can therefore be used as a biomarker for CRC detection in tumor samples and blood or feces, . The most widely used biomarkers for CRC at present are the presence of MSI and KRAS mutations in tumor samples which are used to classify tumors, make disease prognosis and administer a therapy. Although other biomarkers, including determination of FOBT and CEA, have also been used for making disease prognosis, they tend to have high specificity but very low sensitivity (7, 8).

On the other hand, MLH1 gene is known as the mismatch repair (MMR) gene that provides instructions for making proteins that are crucial in DNA repair. The MLH1 protein in combination with another protein, PMS2 (produced from the PMS2 gene), form a two-protein complex called a dimer. There are seven MMR DNA proteins in humans namely MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2 (9).

Another gen TP53, provides instructions for making tumor su ressor proteins. p53 functions not only as a negative regulator of the cell cycle but also in the cell's response to DNA damage or s ss. Cellular p53 protein levels and function are regulated by another e product namely the mouse double minute 2 oncogene (MDM2) as a negative regulator. MDM2 is a transcriptional target of p53, and expression is induced by the binding of p53 to an internal promoter in the MDM2 gene that can se mutations (10).

Most TP53 mutations change a single amino acid in the protein that lead to the production of an altered protein version that can no longercontrol cell proliferation and cannot induce cell apoptosis in cells accumulated with mutated or damaged DNA. As the consequences, tumor cells grow followinguncontrolled cell divisio .

In this case, Gene Mismatch Repair (MMR) plays an important role in the recognition and error repair after replication to prepare for cell division. The errors accumulation in repeated DNA fragments lead to mutations in the target gene. Mutations in the mismatch DNA repair gene, i.e., defective MMR DNA system (microsatellite instability) have been reported to be about 20% in the sporadic CRC and Lynch syndrome. Methylation by CpG island methylator (CIMP), a dinucleotide methylation, takes place at the initial transcriptio site upstream of many genes. They are reported to be associated with approximately 15% to 20% of sporadic CRCs. The CpG

island methylator phenotype mainly carries out hypermethylation of the promoter region of any genes. These methylators methylate certain marker genes in the tumor positives.

It is currently understood that MSI and CIN tumors develop through two different pathways in the colon, the sessile serrated pathway induces MSI tumors in the proximal colon, and the traditional adenoma-carcinoma pathway induces CIN tumors in the distal colon. While APC is thought to be the precursor to CIN tumor , BRAF is one of the earliest identified alterations among MSI precursorsDuring CIN tumorigenesis, genetic complexity intensifies due to chromosomal instability, while on the other hand, CIMP leads to epigenetic instability among MSI precursors, ultimately affecting MLH1, as a cause of MSI.

A deficiency in MMR generates an environment in which cells rapidly build up mutations, including those that induce cancer development. The MMR core in humans consists of three heterodimeric protein complexes involved in recognizing MMR . The first protein complex, hMUTSα (consisting of hMSH2 and hMSH6) recognize and preferentially bind single-nucleotide mismatches, while the second, hMUTSβ (hMSH2 and hMSH3) recognize and preferentially bind insertion-deletion loops. After mismatch recognition, the third protein complex, hMUTL, consisting of hMLH1 paired with hPMS2 or hPMS1, is recruited into the lesion where ATPase and enzymatic endonuclease activity are engaged to complete the repair process. TP53 mutations in CRC were found to be less common in MSI tumors (10–20% of cases) than in CIN tumors (~50–60% of cases) which suggested that MMR deficiency does not cause TP53 mutations in MSI tumorigenesis. Then loss of heterozygosity (LOH) can occur if the wild-type allele remains or the short arm deletion of chromosome 17 is present. In contrast, copy-neutral LOH (cnLOH) occurs when there is no net change in the copy number of the affected allele (11).

MSI has been used as a clinical tool to diagnose tumor. Microsatellite repeats specific for MSI can be detected by a relatively simple PCR amplification. MSI can also be detected by comparing the length of nucleotide repeats in tumor cells and adjacent normal cells using using fluorescent primersin RT-PCR (Real Time Polymerase Chain reaction)coupled with capillary electrophoresis which was the approach in this study. PCR is a commonly used detection method, using primers that amplify target DNA fragments *in vitro* (12). This study was aimed at determining the expression of hMLH1 and p53 mRNA genes in the feces of patients with colorectal carcinoma in various stages and grades of tumors using RT-PCR. This study is an observational study with a cross-sectional approach on the feces of patients with colorectal carcinoma regardless of the stage of the disease.

2. Materials and Methods

Our work was fully compliant with the STROCSS criteria and has been reported with the checklist completed (13). Also, our work has been registered with a unique identifying number (UIN): Researchregistry7319 (https://www.researchregistry.com/browse-the-registry#home/registrationdetails/617ed3cb0ec644001e8e6300/).

Ethical approval has been approved by ethics commission of Faculty of Medicine, Hasanuddin University reference no. 798/UN4.6.4.5.31/PP36/2020.

2.1. Diagnosis and determination of tumor staging

The diagnosis of carcinoma was preliminary established based on histopathological examination of the tumor mass sample through endoscopy or surgery.

Tumor staging was determined by the results of intraoperative findings, including tumor extension, lymph node involvement, and metastasis. The tumor mass substrate was adjusted according to the topography of the colon and rectum. The research material was taken through the

Digital Rectal Examination (DRE) or directly from the patients' feces.

The inclusion criteria of research subjects to maintain objectivity of the samples were CRC patients based on histopathological examination results as adenocarcinoma in various stages and grades. Exclusion criteria for research subjects were CRC patients who were on neo-adjuvant chemotherapy and/or radiotherapy, which could affect the expression of the hMLH1 and p53 genes. The CRC stage was determined by the TNM system and histopathological staging. CRC was graded based on the histopathological examination results.

2.2. Selection of samples and RT-PCR technique

The sample was an affordable population that meets the research criteria and expresses their willingness to participate in the study in writing by signing the consent form to participate in the study. Samples was taken from CRC patients in Siloam Hospitals and Dr. Wahidin Sudirohusodo hospital located in Cikarang, Bekasi and Makassar, Indonesia, respectively. Samples were collected from December 2020 to May 2021.

Adequate sample size according to the Harry King nomogram is 41, however in order to anticipate possible dropout of respondents, an extra 10% was added making a total of 48 respondents. We did not categorized the samples in two groups. No division of intervention between groups was applied. All samples were treated as one.

Feces were sampled from patients with CRC using the DRE method or directly from the patients' feces. We used100 μg/μl feces per sample. Initially, debris and colonocytes in the feces were separated by centrifuging at 1000rpm for two min. Then DNA extraction was performed. Real-time PCR was then carried out to identify the mRNA expression profile of the two target genes, namely hMLH1 and p53.

2.3. Data analysis

The data obtained were analyzed by using the hMLH1 and p53 mRNA gene expression test according to tumor stage and grading, which consisted of data homogeneity test (Levene statistic), data normality test using the Shapiro-Wilk test, and continued with the One-way ANOVA test followed by with LSD Test to group non-significantly different samples.

3. Results

The topographic features of the CRC substrates were 35% the rectum, followed by 31% the sigmoid colon, 21% ascending colon, 6% transverse colon, and 6% descending colon. Most of the tumor were of stage three tumor (35%), followed by stage 2 (33%) and stage 4 tumors (31%). Stage 1 tumor was not found in the samples.

The tumor grade was dominated by Well Differentiation grade 1 (60%), then Moderate Differentiation grade 2 (27%), and Poorly Differentiation grade 3 (13%). All adenocarcinoma fecal samples showed overexpression of mRNA (more than twice the normal amount in Fold Change units) of hMLH1 and p53 genes in various stages (stages 2,3,4) and tumor grades (G1, G2, G3). The mRNA expression of the hMLH1 gene with the highest average value was in grade 1 (Mean: 8.66), the lowest was grade 3 (Mean: 7.88), the mRNA expression of the p53 gene with the highest average value was in grade 3 (Mean: 10.32) and the lowest was grade 1 (Mean: 9.22) (Table 1, Fig.1

The mRNA expression of the hMLH1 gene with the highest average value was at stage 2 (Mean: 9.59875), the lowest was stage 4 (Mean: 7.19440), the mRNA expression of the p53 gene with the highest average value was at stage 4 (Mean: 11.68887) and the lowest was stage 2 (Mean: 7.40544) (Table 2., Fig.2.).

The mRNA expression data for the hMLH1 and p53 genes respectively had values of 0.121 and 0.289 (>0.05), which means all data were homogeneous. The Shapiro-Wilk test showed that the data were normally distributed (sig > 0.05). The results of the one-way Anova test showed a significant difference in the mRNA expression data for the hMLH1 and p53 genes (sig. <0.05). The LSD test showed significant differences in the mRNA expression values of the hMLH1 and p53 genes between all different stages of colorectal cancer.

The mRNA expression data for the hMLH1 and p53 genes had values > 0.05, meaning that all data were homogeneous. The data were normally distributed (sig > 0.05). The results of the one-way Anova test showed that there was no significant difference in the expression data for hMLH1 and p53 (sig.>0.05), further LSD test was not performed (Fig.1.).

4. Discussion

This study suggested that the RT-PCR is highly sensitive and specific for detecting CRC at an early stage, allowing patients to be treated before they reach more severe or metastatic disease stages. Preventive screening of at-risk populations is possible using this relatively rapid technique, which can reduce mortality and morbidity rates of CRC (12).

The results of statistical analysis showed significant differences in the mRNA expression values of the hMLH1 and p53 genes among all different CRC stages. Although the results obtained were significantly different, the mean value of p53 gene mRNA expression was not directly proportional to the stage it affected. At stage two, the mRNA expression of the p53 gene was lower than that of stage four. This was different from the mean value of mRNA expression of the hMLH1 gene which was directly proportional to the stage, where the mRNA expression value of the hMLH1 gene was higher in stage two compared to the lower expression value in stage four. However, the CRC grade for the mRNA expression of the hMLH1 and p53 genes showed no significant difference.

Tumor suppressor gene which is referred as "tumor suppressor gene", or by its synonym "anti-oncogene", or "recessive oncogene" is a term used to describe genes that can inhibit tumor growth. The hMLH1 and p53 genes are genes that belong to this group. Multistep carcinogenesis requires the action of activated oncogenes and the inactivation of tumor suppressor genes (14).Both the tumor suppressor and onco genes are believed to play a critical role in the initiation and development of most neoplasms, including CRC. The expression of p53 mRNA gene in our study contradicted a tumor suppressor gene pattern but coincided with that of oncogene. We speculated that this change of pattern was likely due to mutation, as this phenomenon has been observed by other researchers in tumor cells. hanges in p53 expression have been described in between 44% (15) and 55% of colon tumors. p53 gene mutations in two tumors associated with increased mRNA expression have been reported (16). Remvikos *et al*. (1990) found significant associations between the increase of p53 and the presence of DNA aneuploidy (17). In rectal carcinoma, p53 mutation and loss of the allele on the short arm of chromosome 17 appears to cause genetic changes that deactivate the function of gene p53 as the tumor suppressor, that the changes in this gene cause the p53 protein to malfunction (18).

Previously p53 was reported in many nonviral altered tumor cells, but not in normal cells, and is therefore called a cellular tumor antigen. However, with better detection methods, p53 was also found in normal cells, although in very small levels. Research by Mercer *et al*. showed that p53 expression is required for resting cell transit from G0 to G1 (14). Transfection of p53 into primary cells led to the immortalization of these cells, while cotransfection of p53 with activated ras oncogene caused the conversion of cells to completely alter the phenotype. Furthermore, transfection vector of p53 expression into various cells suggested that the effect of overexpressed p53 was related to cell transformation or tumorigenesis (14) The overexpression of p53 in tumor cells was most often due to the metabolic stabilization of the p53 protein at the post-translational level. This leads to transformed cells containing an approximately 100-fold increase in p53. An increase in the amount of p53 in tumor cells will propel these cells from one cycle of the cell cycle to the next, which will lead to increased proliferation.

P53 mutations are the most frequently detected genetic changes in human cancers found in more than 50% of all human cancers. The p53 gene mutation alters the conformation of the nuclear protein product, which can inactivate the wild-type p53 protein. Elevated p53 protein expression or inactivation mutations of the p53 gene have been demonstrated in various malignant tumors in humans including carcinomas of the colon and rectum, breast, prostate, lung, stomach, thyroid,

and liver. In colorectal carcinoma, the occurrence of p53 mutations varies and is found in 50% to 70% of cases.

Based on the histological assessment of colorectal adenocarcinoma, the results of the study conducted by Khudier et al showed that 41.2% were well-differentiated, 47.1% were moderately differentiated, and 11.8% were poorly differentiated. While the study was conducted by Valera et al showed that 57.6% were well-differentiated, 39.6% were moderately differentiated, and 2.8% were poorly differentiated.

In their study, there was no relationship between CRC stage and p53 expression which was in line with the results of research conducted by Demirbas and colleagues who also did not find a significant relationship between p53 and stage but did not agree with the results of the research conducted by Jackson and colleagues who found a significant relationship between p53 and CRC stage (19).

Evidence of p53 mutations is found in most CRCs in the clinical population indicating that chromosomal instability is responsible for the majority of colorectal carcinomas. No association could be found between p53 expression and histologic type, histological grade, and stage (19).

The MLH1 gene provides instructions for making proteins that play an important role in repairing errors made during DNA replication in preparation for cell division. The MLH1 protein combines with another protein called PMS2 to form a protein complex. Repair is done by removing the part of the DNA that contains errors and replacing the part with the corrected DNA sequence. The MLH1 gene is a member of a set of mismatch repair (MMR) genes. MMR deficiency is associated with hereditary non-polyposis colorectal cancer (Lynch syndrome) and sporadic cases of CRC. This MMR system is well known for maintaining the overall stability of the genetic material, deficient cells exhibit a mutator phenotype with a high rate of microsatellite mutations.

5. Conclusions

This study showed that there was a relation between the hMLH1 mRNA gene expression and the stage of CRC, the higher the value of fold change expression, the lower the stage of CRC. On the other hand, negative correlation was shown between the p53 mRNA gene expression and the CRC stages, the higher the value of fold change expression, the higher the stage of CRC. It was concluded that the hMLH1 and p53 genes in respondents' feces with CRC were overexpressed, but the expression value did not suggest the stages of CRC. hMLH1 mRNA gene expression is in accordance with its function, however p53 gene had an altered expression pattern which might be due to mutation.

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Table 1. Correlation between mRNA expression of the hMLH1 and p53 genes and preliminary grading results

		N	mean	Std Deviation	Std. Error	95% Confidence Interval for mean		min	max
						Lower Bound	Upper Bound		
Expression mRNA hMLH1	Grade1	29	8.66369	1.213515	0.225344	8.20209	9.12529	6,399	10,607
	Grade2	13	8.23815	1.435497	0.398135	7.37069	9.10562	6.168	10.282
	Grade3	6	7.88483	1.522059	0.621378	6.28753	9.48214	6.411	10,607
Total		48	8.45108	1.315768	0.189915	8.06902	8.83314	6.168	10,607
Expression mRNA. p53.	Grade1	29	9.21800	2.286227	0.424542	8.34837	10.08763	5,720	13,880
	Grade2	13	10.12615	2.239878	0.621230	8.77261	11.47970	6.268	13.013
	Grade3	6	10.32367	2.820682	1.151539	7.36354	13.28379	5.138	13,349
Total		48	9.60217	2.339803	0.337721	8.92276	10.28157	5.138	13,880

Table 2. Correlation between mRNA expression of the hMLH1 and p53 genes and preliminary staging results

mRNA expression Gene	Stage	N	mean	Std. Deviation	Std. Error	95% Confidence Intervals for Mean		min	max
						Lower Bound	Upper Bound		
hMLH1	2	16	9.59875	0.551687	0.137922	9.30478	9.89272	8,663	10.362
	3	17	8.48124	1.236147	0.299810	7.84567	9.11680	6.168	10,607
	4	15	7.19440	0.737266	0.190361	6.78612	7.60268	6.218	8,779
	Total	48	8.45160	1.316044	0.189955	8.06946	8.83374	6.168	10,607
p53	2	16	7.40544	1.118007	0.279502	6.80969	8.00118	6.051	9.376
	3	17	9.82847	2.059278	0.499448	8.76969	10.88725	5.138	12.656
	4	15	11.68887	1.396820	0.360657	10.91533	12.46240	8,562	13,880
	Total	48	9.60217	2.339803	0.337721	8.92276	10.28157	5.138	13,880

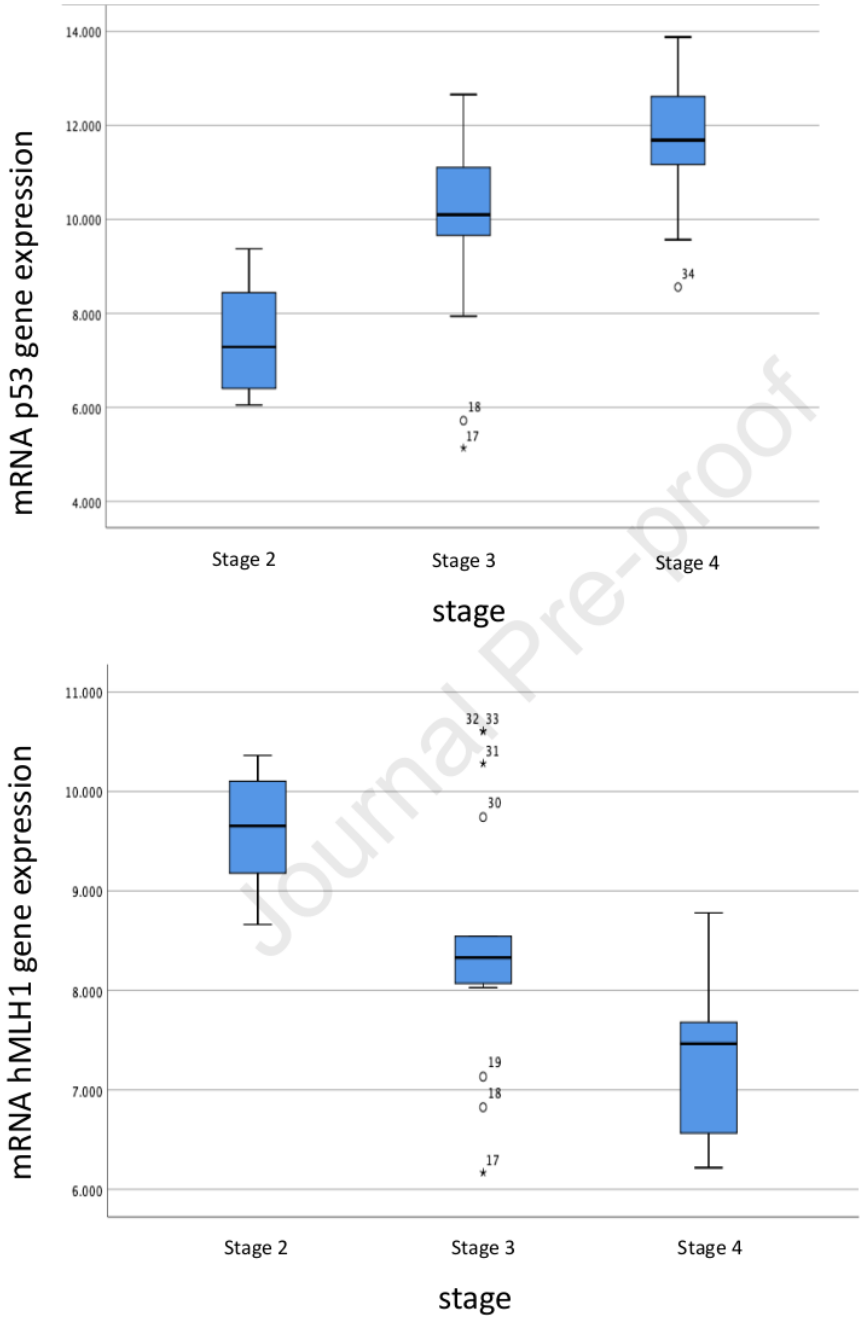

Fig.1. hMLH1 and p53 mRNA gene variables as measured by tumor stage

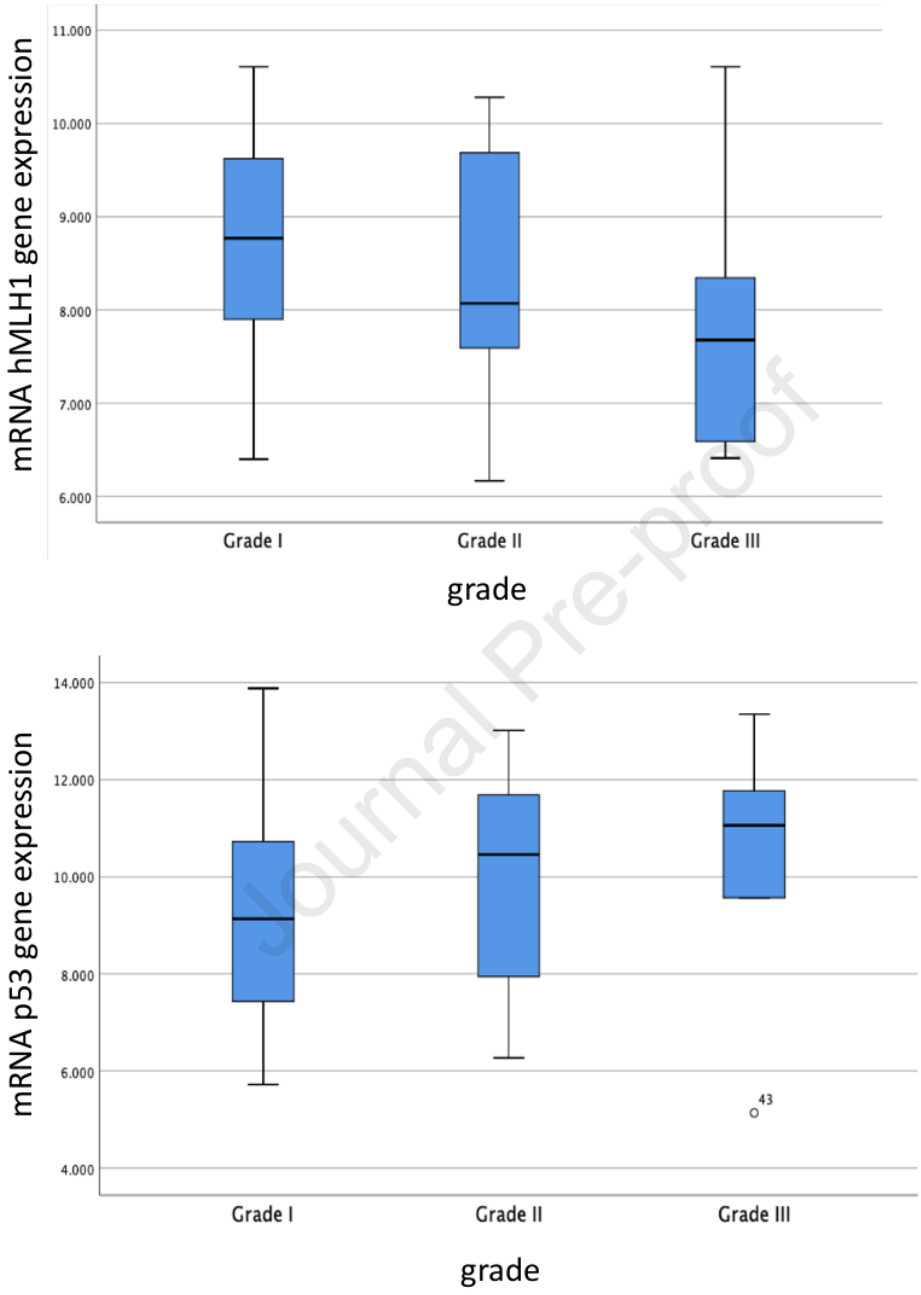

Fig.2. hMLH1 and p53 mRNA gene variables as measured by tumor grade

Highlights:

1. RELATION OF mRNA EXPRESSION OF hMLH1 and p53 GENE IN COLORECTAL CARCINOMA PATIENTS FECES
2. mRNA EXPRESSION IN FECES COLORECTAL CARCINOMA BY RT-PCR EXAMINATION
3. GENE hMLH1 and p53 IN FECES COLORECTAL CARCINOMA PATIENTS

Annals of Medicine and Surgery

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All authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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All sources of funding should be declared as an acknowledgement at the end of the text. Authors should declare the role of study sponsors, if any, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. If the study sponsors had no such involvement, the authors should so state.

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There were no sponsors involvement in the study

Ethical Approval

Research studies involving patients require ethical approval. Please state whether approval has been given, name the relevant ethics committee and the state the reference number for their judgement.

Ethical approval has been approve by ethics commission of Faculty of Medicine, Hasanuddin University reference no. 798/UN4.6.4.5.31/PP36/2020

Consent

Studies on patients or volunteers require ethics committee approval and fully informed written consent which should be documented in the paper.

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Ryanto Karo Karo Sitepu (RKS), Rosdiana Natzir (RN), Warsinggih, (WS), Mochammad Hatta (MH) initiated and designed the study, drafted the manuscript.
All authors have read and approved the final manuscript.

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